Determination of the Viscoelastic Behaviour of Canine Cranial Cruciate Ligaments at Slow Strain Rates.

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Abstract

Background

Viscoelastic behaviour of the canine cranial cruciate ligament (CCL) controls viscous behaviour in the ligament and potentially ligament injury. This study aims to elucidate CCL viscoelasticity at the toe-region of stress-strain curves by investigating ligament response to uniaxial tensile load at slow strain rates.

Methods

Five paired CCLs from skeletally mature and disease free Staffordshire bull terriers were mechanically tested under cyclic load of 10N at three strain rates 0.1, 1 and 10%/min. Using slow strain rates, this study also investigated the effect of altering the order of strain rates during tensile tests such as when strain rate increased by 10% from 0.1 to 10%/min (ascending tests) and decreased by 10% from 10 to 0.1%/min (descending tests).

Results

CCL loading-unloading stress-strain curves at slow strain rates (≤10%/min) showed non-linear behaviour where stresses exponentially increased with increasing strains and CCL stiffness increased with increasing strain rates. The CCL response to the applied load at increasing strain rates from 0.1 to 1 and then to 10%/min was statistically significant ($p<0.05$). Unlike the descending tests, hysteresis showed significant strain rate dependency during ascending tests and decreased by 0.13% and 0.06% with increasing strain rates from 0.1 to 1 and then to 10%/min, respectively.

Conclusions

At slow strain rates, CCLs showed viscoelastic characteristics such as strain rate dependency and hysteresis. Difference in the CCL responses to the ascending and descending tests may be

associated with the strain history of the tissue or high-level of biological variability across samples. Thus, altering test protocols cannot be explicitly linked to mechanical changes within the CCL. However, the changes may be sufficient in a variety of scientific investigations and should be considered when planning experimental studies on history dependent samples.

Keywords

Stifle (knee) joint, canine, cranial cruciate ligament, osteoarthritis, viscoelastic, hysteresis

1 Introduction

Ligaments play a major role in stifle (knee) joint stability [1,2], with primary support being provided by the cranial cruciate ligament (CCL), the caudal cruciate ligament (CaCL), the medial collateral ligament (MCL) and the lateral collateral ligament (LCL) [3,4]. The most commonly ruptured stifle ligament is the cranial cruciate ligament (CCL) [5,6]. Rupture of this ligament is believed to be a leading cause of lameness leading to osteoarthritis [5,7]. The cause of this rupture is often unknown, but factors such as joint conformation, altered ligament extracellular matrix (ECM) composition [8,9], genetics [10] and obesity [11] have been implicated in its aetiopathogenesis. The mechanical behaviour of ligaments is non-linear viscoelastic exhibiting both elastic and viscos behaviour including history- and time-dependent characteristics [12]. The initial part of the non-linear load-deformation behaviour in CCLs is the toe region where ligament fibres tighten and the crimp is removed. In this zone, there is a relatively large deformation of the tissue with little increase in load and this permits initial joint deformations with minimal tissue resistance [13–15]. Typically, higher strain rates will produce stiffer behaviour, producing a higher modulus. This phenomenon of viscoelastic characteristics has been observed in soft biological tissues such as the sclera [16], cornea [17], tendon [18] and ligaments [19]. The tension which develops in a ligament is believed to be dependent on strain rate [20]. For example, slow strain rates result in the development of low tension, whereas high strain rates cause high tension. Several researchers focused on the effect of loading to failure using high strain rates. This is when ligament failure occurrence is likely due to contact or non-contact sport injuries [21–23]. Haut and Little investigated lower strain rates between approximately 2 and 54%/min [24]. In their study on the mechanical properties of the canine CCL, they reported that the tissue stiffness (measured by tangent modulus) increased with strain rate, the overall shape of the stress-strain curve did not undergo major changes, but the transition from the toe region to the elastic region appeared at lower strain levels in tests with higher strain rates. In the same tests, rapid change in the tangent modulus was found with the slow strain rates (between 1.7 and 10.8 %/min) but the change became progressively smaller with higher strain rates (above 10.8 %/min). Moreover, they reported that stress-strain behaviour at the toe region was dependent on strain rates up to 6% strain. Similarly, Pioletti *et al.* showed that for a given strain level the stress increases with augmentation of the strain rate [25]. For example, when they tested bovine ACL at 2400%/min they reported that 70% of stress was due to the effect of strain rate. However, some researchers studied the mechanical properties of the rabbit MCL between the strain rates of 0.66 and 9300%/min and found that the MCL complex was only minimally strain rate sensitive [20,26]. Similarly, others have reported that the strain rate sensitivity decreases with the increase of deformation rate [19,23]. Bonner *et al.* undertook macroscale experiments at strain rates ranging from (~6-300%/min) on the porcine stifle LCL [19]. They observed a typical stress-strain behaviour showing a toe region up to 3-4% strain followed by a linear region. They also believed that at slow strain rate (~6%/min) the unloaded fibrils go through the toe region, where uncrimping of collagen fibres occur, before presenting intra-fibrillar gliding. However, at fast strain rates (~300%/min) fibrils start from an unloaded state then move directly to intra-fibrillar gliding. This review clearly highlights debated findings and limited understanding on the strain rate sensitivity of ligaments in general and CCLs in particular and there is no clear methodological investigations on the effect of slow strain rates to the mechanical response of the CCLs.

Another viscoelastic property of the CCLs is hysteresis, which represents the loss of energy (energy dissipated) within the tissue during loading cycles [12]. Fung thought that this phenomenon is only weakly dependent on strain rates within soft biological tissues [12]. However, Haslach pointed out that Fung's belief in this phenomenon was based on a small number of experiments on rabbit papillary muscle using only three different strain rates [27].

Hence, Fung's findings only approximately support the independence of hysteresis from strain rates. Boyce studied the viscoelastic tensile response of bovine cornea and supported Haslach's suggestion and they found an increase in hysteresis with decreasing strain rates [28]. Current research on hysteresis in ligaments is limited with no studies investigating hysteresis behaviour in canine CCLs, hence limiting our understanding on one of the viscoelastic properties of the CCLs due to slow strain rates at the toe region.

It is the hypothesis of this study that the CCL will show clear viscoelastic properties such as strain rate sensitivity and hysteresis characteristic at the toe region, before the ligament fibres tighten and the crimp in collagen fibres is removed, during the application of slow strain rates (≤ 10%/min). Using slow strain rates, this study also investigates the effect of altering the order of strain rates such as ascending and descending strain rates on the mechanical behaviour of the CCLs.

2 Material and Methods

2.1 CCL storage and preparation

Cadaveric disease-free stifle joints from five skeletally mature Staffordshire bull terrier canines euthanatized for reasons other than musculoskeletal injury were obtained with full ethical permission from the Veterinary Research Ethics Committee (School of Veterinary Science, University of Liverpool (VREC65)). Stifle joints with body mass >15kg and age between 1.5 and 5.0 years were tested in this study. The entire stifle joints were frozen at -20°C until required and defrosted at room temperature prior to removing the CCLs as a femur-ligament-tibia complex, Figure 1a, b. Firstly, the stifle joint was dissected, and the CCLs were extracted in a manner so that there was no damage to the ligament origin or insertion site. Approximately 10mm of the femoral and tibial bones were left connected to the ligament to form a femur-

CCL-tibia complex, which allowed for the measurement of end-to-end ligament deformation as well as helping to facilitate the clamping of the specimen.

The dissected femur-CCL-tibia complexes were wrapped in paper soaked with phosphate buffered saline (PBS) and frozen at -80°C until they were required for testing [29]. Immediately prior to testing, the samples were thawed at room temperature and two 1.1mm arthrodesis wires (Veterinary Instrumentation, Sheffield, UK) were drilled through the tibial and femoral bone ends, Figure 1c. These pins were placed in order to provide extra grip as well as to replicate the ligament's normal physiological position before being secured using custom built steel clamps, Figure 2. The clamps were designed to provide a secure grip as well as ensuring that ligaments were free and unobstructed throughout the experiment. The clamped samples were then mounted on an Instron 3366 materials testing machine (Instron, Norwood, MA) fitted with a 10N load cell (Instron 2530-428 with 0.025 N accuracy) to perform mechanical testing.

2.2 CCL Length

The length measurement protocol adopted in this study was based on a method described in other studies, which determined the average length of CCL from the craniomedial and caudolateral portions of a ligament [9,30]. However, this methodology was improved in the current study by taking measurements from the cranial and caudal faces of the CCL, as well as the lateral and medial faces. The mean values of these four length measurements were recorded to give an accurate record of the length of the CCL before deformation. CCL length was measured between the femoral and tibial attachments with Vernier callipers (±10μm).

2.3 CCL cross-sectional area (CSA)

A modified method of Goodship and Birch was used to measure cross sectional area (CSA) of the CCLs [31]. Alginate dental impression paste (UnoDent, UnoDent Ltd., UK) was used to make a mould around the CCL. Two different approaches were used to make the moulds: hanged and non-hanged methods. The non-hanged method (Figure 3a) involved laying the sample flat on a bench before clamping and applying the paste. The hanged method (Figure 3b) involved the sample being clamped and mounted on an Instron 3366 materials testing machine prior to paste application. Once set, the alginate paste was sliced with a blade and removed from the CCL. CCL replicas were then made by injecting polymethylmethacrylate (PMMA) paste into the hardened alginate moulds. Once the PMMA was set, it was then removed from the mould and cut into two in the middle to measure midpoint CSA. Image J (a public domain Java image processing program) was used to determine CSA at the midpoint of the ligament (Figure 3c).

2.4 Testing protocol

A preload of 0.1N was applied to remove laxity within the CCL [32]. This was followed by preconditioning the CCLs to ensure that they were in a steady state and would produce comparable and reproducible load-elongation curves [12,33,34]. Preconditioning involved performing ten loading-unloading cycles up to a maximum load of 10N at 10%/min strain rate [35]. Subsequently the CCL was subjected to cyclic tensile loading-unloading tests investigating stress-strain behaviour of the ligament at the toe region through the application of 10N load at sequential slow strain rates of 0.1, 1 and 10%/min. Each strain rate consisted of three loading-unloading cycles which allowed for reproducible results. Between each two cycles, including the preconditioning procedure, a period of six minutes recovery time was given [36]. This time had been identified by analysing the load-deflection curve data and was observed to be sufficient for the CCL to closely reach its original state. Left CCLs were exposed to an ascending strain rate test in which the rate of strain was increased from 0.1 to 1 and to 10%/min and right CCLs were exposed to a descending strain rate in which the CCL was tested under a decreasing strain rate from 10 to 1 and to 0.1%/min [25,37].

2.5 Mechanical properties

Similar to Dorlot *et al.*, load-elongation data was collected to study the mechanical properties of the canine CCLs [38] and then analysed using Excel spreadsheets (Microsoft Office 2010, US) and MATLAB (MATLAB2015a, The MathWorks, Natick, US). Similar to other studies on canine CCLs [24,26], approximate stress was calculated by dividing the applied load by the CSA of the CCLs at the mid region (Equation 1) and the corresponding elongation data was used to calculate strain in the tissue (Equation 2). Subsequently, exponential curves were fitted onto the calculated stress and strain data using the least squares method (Equation 3). Fitting an exponential curve to the stress-strain behaviour of soft biological tissue such as the mesentery of rabbits was proposed by Fung [39] and the exponential equation was further utilised on canine CCLs by Haut and Little [24].

Similar to the methods used in the study of soft tissue by Geraghty *et al.*, the tangent modulus of the CCLs was calculated by applying Equation 4 and the polynomial curve fit, using the least squares method, was employed to produce tangent modulus-stress curves (Equation 5) [40]. Hysteresis, another viscoelastic property of soft tissues defined as the area between loading-unloading stress-strain curves, was calculated using numerical integration [41].

$$\sigma = \frac{F}{CSA} \qquad \text{Equation 1}$$

where σ is stress in MPa, F is applied load in N and CSA is cross-sectional area in mm^2.

$$\varepsilon = \frac{\Delta L}{L_0} \qquad \text{Equation 2}$$

where ε is strain, ΔL is change in length in mm ($\Delta L = L_0 - L_1$), and L_0 is initial length of the ligament in mm.

$$\sigma = a(e^{b\varepsilon} - 1) \qquad \text{Equation 3}$$

where a and b are constants, whose values are determined from fitting the exponential equation to the experimental stress and strain values..

$$E_{tan} = \frac{\sigma}{\varepsilon} \qquad \text{Equation 4}$$

where E_{tan} is tangent modulus in MPa.

$$E_{tan} = ax^3 + bx^2 + cx + d \qquad \text{Equation 5}$$

where $a, b, c,$ and d are coefficients.

2.6 Statistical Analysis

CCL lengths measured at different planes were categorised into cranial, caudal, medial and lateral groups. Statistical tests were performed using one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test for multiple comparisons. Analyses were performed in Microsoft Office Excel and $p<0.05$ was an indication of statistical significance.

CSA measurements obtained from the hanged and non-hanged methods were statistically analysed using a two-tailed t-Test in Microsoft Office Excel studying statistical differences between CSA measurements from the two methods. Statistical significance was set at $p<0.05$.

CCLs collected for the ascending and descending strain rate protocols were statistically compared for their significant differences in hysteresis, area under stress-strain and tangent modulus-stress curves using a two-tailed t-Test. One-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons were performed in Microsoft Office Excel to analyse differences between area under the stress-strain, tangent modulus-stress and hysteresis curves at the three strain rates (0.1, 1 and 10%/min). This statistical test was performed twice and independently, once to test CCL behaviour during the ascending strain rate tests and another time during the descending strain rate tests.

3 Results

3.1 CCL Samples

The CCL samples (n=5 paired stifle joints) used to investigate mechanical properties of the ligament were of mixed gender (female=3 and male=2) and had a body mass of 21.46±3.75 kg with body condition scores of 3.5 ± 0.94.

3.2 CCL length

The lengths of CCLs at the cranial, caudal, medial and lateral planes are presented in Table 1 and the average length values in these planes were used in the calculation of the material properties of the CCLs. The ANOVA test showed statistically significant results ($p<0.05$) in measuring CCL length in different plane views. The post-hoc results showed significant differences ($p<0.05$) between cranial and caudal, cranial and lateral, and caudal and medial planes (Supplementary Materials (Table 1)).

Table 1: Length of CCLs (mm) at different measurement planes.

CCL Sample Number	Cranial Plane		Caudal Plane		Medial Plane		Lateral Plane		Average		Standard Deviation (SD)	
	Right	**Left**	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**
1	13.51	14.54	7.88	8.16	11.76	14.10	11.00	12.31	11.04	12.28	±2.35	±2.91
2	22.79	22.07	11.20	12.00	17.00	13.1	20.54	16.93	17.88	16.03	±5.05	±4.55
3	21.44	23.16	13.53	11.55	17.51	19.05	16.05	14.37	17.13	17.033	±3.31	±5.12
4	17.88	18.58	10.37	13.02	13.94	15.12	16.51	16.82	14.68	15.89	±3.30	±2.38
5	15.30	17.83	9.20	9.38	13.50	15.81	13.1	12.31	12.78	13.83	±2.57	±3.74
Mean ± SD	**18.71 ± 3.54**		**10.63 ± 1.96**		**15.09 ± 2.25**		**14.99 ± 2.90**		**14.86 ± 2.31**		**-**	

3.3 CCL cross-sectional area (CSA)

The average CSA value obtained through the non-hanged method was 9.48% smaller than CSA value in the hanged method. CSA of CCL determined from the non-hanged was 15.30±3.09 mm^2 and the hanged method was 16.75±4.58 mm^2. However, statistical analysis confirmed that there was no significant difference between the two methods ($p=0.42$). The CSA of each individual CCL can be found in Supplementary Materials (Table 2).

3.4 Mechanical properties

3.4.1 Stress-strain

The loading (Figure 4a, c) and unloading (Figure 4b, d) stress-strain curves at 0.1, 1 and 10%/min strain rates conformed to the typical non-linear behaviour as expected in ligament tissue [24] for all five CCLs and for both testing protocols (ascending and descending tests). The loading and unloading stress-strain curves illustrated an exponential growth with increasing strain level, and a more pronounced stiffness was observed with increasing strain rates.

During the ascending testing protocol, the stress resulting from a 0.03 mm/mm strain (approximately at the transition region from non-linear to linear stress-strain curves) increased by 26.5±0.11% at 1%/min and 35.0±0.12% at 10%/min when compared to stress generated by the same amount of strain at 0.1%/min. Similarly, the stress-strain curves at lower (<0.03 mm/mm) and higher (>0.03 mm/mm) strain levels showed a similar increase in stress with increasing strain rates (Figure 4a).

During the descending testing protocol, the stress resulting from a 0.03 mm/mm strain increased by 0.40±0.35% at 1%/min and 3.80±0.38% at 10%/min when compared to stress due to the same amount of strain at 0.1%/min. Similarly, the stress-strain curves at higher strain levels (>0.03 mm/mm) produced a similar increase in stress with increasing strain rates. However, the lower strain levels (<0.03 mm/mm) showed inconsistent results. For instance, at a 0.01 mm/mm strain level the stress decreased by 4.70±0.02% at 1%/min and 5.20±0.03% at 10%/min when compared to stress at the same strain level at a 0.1%/min strain rate (Figure 4c).

Stress-strain data collected from ascending and descending testing procedures demonstrated similar trend lines, Figure 5. The ascending stress-strain curves deviate from the descending curves at approximately 0.03 mm/mm strain level. There were no significant differences between the two test protocols at 1%/min ($p=0.104$). However, stress-strain behaviour during the ascending tests was statistically different from the descending tests at 0.1%/min ($p=0.007$) and 10%/min ($p=0.020$).

The response of the CCLs to the tensile load at different strain rates presented statistically different behaviour in both ascending (*$p<0.01$*) and descending tests (*$p=0.01$*). The statistical tests showed statistically different stress-strain curves during ascending tests between 0.1 and 1%/min (*$p=0.01$*), 0.1 and 10%/min (*$p<0.01$*), 1 and 10%/min (*$p<0.01$*), whereas during descending tests the tensile response was statistically different only between 0.1 and 10%/min (*$p=0.01$*).

3.4.2 Tangent modulus-stress

Tangent modulus (E_t), indicating the stiffness behaviour of the CCLs, increased with increasing stress and strain rate (Figure 6a, b) in both ascending and descending tests. This increase in tangent modulus with stress was evidenced during both loading and unloading. The increase in stiffness of the CCLs in ascending tests with increasing strain rates was different compared to the descending test, such that in descending tests there was inconsistency in tensile response below 0.15 MPa. In addition, there were greater variations in the CCLs' responses to tensile loading during descending compared to ascending test protocols. However, statistical analysis showed no statistically significant difference in tangent modulus between the two protocols.

Tangent modulus-stress curves at 0.1, 1 and 10%/min strain rates were normalised by the tangent modulus-stress curve at 0.1%/min (Figure 6c, d). The normalised tangent modulus-stress curves (E_t / $E_{t\,(\dot{\varepsilon} = 0.1\%/\mathrm{min})}$) were plotted to describe the amount of increase in stiffness with increasing strain rates. The increase in stress from 0.2 to 0.5 MPa introduced stiffer behaviour by a factor of approximately 0.998 to 1.036 at 1%/min and 1.001 to 1.039 at 10%/min in ascending tests. However, the corresponding values in descending tests were a factor of approximately 1.001 to 1.039 at 1%/min and 1.005 to 1.066 at 10%/min (Figure 6c, d). Stiffness behaviour of the CCLs during ascending and descending tests was statistically proven to be strain rate dependent. The statistical difference was significant between 0.1 and

1%/min strain rates (*p=0.02* for ascending and *p=0.03* for descending) and between 0.1 and 10%/min (*p=0.01* for ascending and *p<0.01* for descending). However, no statistically significant changes in tangent modulus curves were noted when comparing the stiffness curves at 1 and 10%/min (*p=0.67* for ascending and *p=0.13* for descending).

3.4.3 Hysteresis

Increasing strain rate from 0.1 to 1 and then to 10%/min during the ascending test protocol and decreasing strain rates from 10 to 1 and then to 0.1%/min presented similar characteristics for hysteresis. The hysteresis was smaller at higher strain rates compared to hysteresis at lower strain rates (Figure 7). This decrease in hysteresis with increasing strain rate was statistically significant between 0.1 and 1%/min (*p=0.030*), and 0.1 and 10%/min (*p=0.002*) in ascending tests, however, no statistically significant changes were found in hysteresis between 1 and 10%/min (*p=0.581*). In descending tests, hysteresis was not strain rate sensitive (*p=0.497*), and no statistical differences were found in hysteresis between ascending and descending tests (*p=0.077*).

4 Discussion

The aim of this study is to gain a greater understanding of the viscoelastic behaviour of the canine CCLs at the toe region of the stress-strain curves under slow strain rates. Hence, experimental study was carried out to investigate the nonlinear viscoelastic properties of CCLs, such as stress-strain and hysteresis behaviour, from healthy canine stifle joints at slow strain rates (≤10%/min). The findings in this study are the first to report the slow strain rate dependency of the canine CCL across three orders of magnitude with ascending and descending test arrangements. Haut and Little showed that with high strain rates, the toe region of stress-strain curves appears at lower strain levels [24]; therefore, it was the objective of the current study to focus on the stress-strain behaviour at the toe-region (low strain level) and utilise slow strain rates (≤10%/min) at different magnitudes such as 0.1, 1, and 10%/min. In the current study, the shape of the stress-strain curves follows a similar pattern to that previously found in biological tissues such as tendons and ligaments [19,23–25,37]. The tensile response of the CCL during ascending and descending tests was found to be significantly different ($p<0.05$), such that during the descending tests the CCL behaved more stiffly and inconsistently at low strain level. This finding of a change in tissue behaviour with altering strain rate orders during tensile tests is not in agreement with the findings previously reported [25,37]. For example, Pioletti *et al.* loaded bovine ACLs up to 300N with seven different strain rates (6, 60, 300, 600, 1200, 1800 and 2400 %/min) and then tested for strain rate order by reloading the ACLs at the 6 and 300%/min strain rates [25]. However, they found identical stress-strain behaviour for the initial and reloaded ACLs. It is important to note that these studies applied higher strain rates (6-2400%/min) than those used in the current study and they reloaded the tissue in an ascending strain rate order only [25,37]. Therefore, it is possible that the effect of the change in strain rate order is more pronounced at slower strain rates (≤10%/min) because the unloaded fibrils go through the toe region and show intra-fibrillar gliding [19]. However, at fast strain rates (≥300%/min) fibrils go from an unloaded state directly to intra-fibrillar gliding where the matrix bond between the collagen molecules are broken before the removal of collagen crimps [19]. Moreover, the strain rate sensitivity of the CCLs was more pronounced and statistically significant ($p<0.05$) during the ascending than the descending test procedure.

CCL stiffness (tangent modulus) during loading and unloading cycles in the ascending and descending test procedure increased with strain rate. Although the tangent modulus-stress curves were different in the ascending and descending tests, this difference was not statistically different. In both test procedures, the canine CCL was stiffer during unloading than loading cycles. This resulted in considerable hysteresis (energy dissipation) between the loading and unloading stress-strain curves. The stiffer behaviour results in less dissipated energy in the loading and unloading stress-strain curves and hence the magnitude of hysteresis decreased with increasing strain rates. This phenomenon was observed during both ascending and descending tests. Hysteresis was found to be strain rate sensitive during the ascending test in each sample tested which reflects the findings of other authors [27,28]. During descending tests, the CCLs were not strain rate sensitive, and hysteresis was independent from strain rate. Previous studies have shown that if a soft tissue is not sensitive to strain rate, the hysteresis is expected to be relatively uniform with respect to strain rates [26]. The CCL is a history and time dependent biological tissue; therefore, strain history might have caused different CCL behaviour during ascending and descending tests. One of the factors that could affect strain history in soft biological tissues is insufficient recovery time during loading-unloading cycles. In this study, recovery procedures reported in previous literature were followed allowing adequate recovery time between loading and unloading cycles [24,42,43]. It is widely known that a higher strain rate results in the development of a high stress in ligaments [19,20,24], hence a longer time might be needed to allow for the uncrimped collagen fibres to return to the crimped state. However, a lower strain rate is likely to develop a lower stress in ligaments, hence a shorter time is required to allow for the uncrimped collagen fibres to go back to the normal state [25]. Therefore, it is possible for the CCL to behave in a similar fashion during both ascending and descending tests if a longer time is provided for tissue recovery from stresses caused by a higher than a lower strain rate.

The current study may be limited by disregarding the complexity of the anatomical structure of the CCLs which consists of two fibre bundles (caudolateral (CLB) and craniomedial bands (CMB)) functioning independently from one another in stifle joint flexion and extension [3,44]. Independent functioning of the CLB and the CMB allows the fibre bundles to recruit to their maximum potential. However, it is important to note that these two fibre bundles are not structurally segregated on tissue scale, thus allowing the ligament to function as a one united tissue. Future studies could investigate the application of a non-contact measuring method to optically capture local strain on the surface of the CCLs during different loading conditions.

Further limitations may be observed due to varying cadaveric properties such as gender, age, bodyweight and body condition, some of which is known to affect the mechanical responses of the ligaments [11,20,45]. Due to a low sample number, this study was unable to separate the CCLs by gender, age, bodyweight and body condition for statistical analyses, although high standard deviations may be evident due to differences in cadaveric properties of the ligaments. Future studies should aim to include a larger sample number.

With these considerations in mind, future research could aim to accurately assess non-linear viscoelastic behaviour across the surface of the CCL by employing imagery approaches such as digital image correlation methods and taking varying cadaveric properties into account by increasing the sample size.

5 Conclusions

- The current study is the first to focus on the viscoelastic behaviour, such as strain rate sensitivity and hysteresis, of canine CCLs at low level strain to better understand the tissue behaviour at the toe region which is important because at this region ECM components such as PGs dominate tissue response. It is likely that CCLs displayed an initial low stiffness at low strain rates due to uncrimping of collagen fibres and the contribution of other components in the ECM.
- Arranging mechanical tests in different orders of strain rates showed different results, such that tensile responses of the CCL during the ascending tests were

significantly different from the descending tests. The strain rate sensitivity of the CCLs was statistically significant during ascending tests only.

- The stress-strain behaviour of the CCLs was stiffer during unloading than loading. This resulted in a considerable amount of dissipated energy between loading and unloading stress-strain curves. In addition, the stiffer behaviour during higher strain rates resulted in less hysteresis. Therefore, hysteresis during the ascending tests was dependent on strain rate as it decreased with increasing strain rates. However, this phenomenon was not statistically significant during the descending tests.
- The different behaviour of the CCLs under tensile tests in the ascending and descending ordering of strain rate may be associated with the strain history of the tissue or high-level of biological variability across samples. Therefore, this study speculates that a longer time may be required for tissue recovery from stresses caused by a higher strain rate. The outcome of this experimental study indicates the need for further investigations on the viscoelastic behaviour of the canine CCLs when loaded with different orders of strain rates.

Declarations of interest

None.

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